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Allelic loss at the short arm of chromosome 11 is one of the most common and potent events in the progression and metastasis of breast cancer. Here, we present evidence that the Integrin-Linked Kinase (*ILK*) gene maps to the commonly deleted chromosome 11p15.5 and suppresses malignant growth of human breast cancer cells both *in vitro* and *in vivo*. *ILK* is expressed in normal breast tissue but not in metastatic breast cancer cell lines or in advanced breast cancers. Transfection of wild-type *ILK* into the MDA-MB-435 mammary carcinoma cells potently suppressed their growth and invasiveness *in vitro*, and reduced the cells' ability to induce tumors and metastasize in athymic mice. Conversely, expression of the ankyrin repeat or catalytic domain mutants of *ILK* failed to suppress the growth of these cells. Growth suppression by *ILK* is not due to apoptosis but is mediated by its ability to block cell cycle progression in the G1 phase. These findings directly demonstrate that *ILK* deficiency facilitates neoplastic growth and suggest a novel role for the *ILK* gene in tumor suppression.

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PI - Signature

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A.   INTRODUCTION

Genetic alterations that occur in breast cancer are believed to be of importance for initiation as well as progression of the disease. These genetic alterations lead to the loss or activation of a number of critical genes, such as those involved in cell proliferation, differentiation, apoptosis, and genetic stability. The genetic abnormalities most frequently observed in breast tumors are amplification of proto-oncogenes (*MYC*, *ERBB2* and *CCND1*), mutations of *TP53*, and loss of heterozygosity (LOH) on chromosomes 3p, 6q, 7q, 8p, 9p, 11, 13q, 17, 18q and 22q (1, 2). Metastatic phenotypes have been linked to such genes as *NME1* (17q), *CDH1* (16q), *BRMS1* (11q), and *KISS1* (1q) (1, 3-5). LOH analyses have defined regions of deletion associated with metastasis on chromosomes 3p21, 15q14, 16q22 and 11p15 (2, 6)

Frequent genetic alterations on chromosome 11p15 suggest a crucial role for this region in breast (6, 7) and other adult (8-12) and childhood cancers (13-17). More recently, we have mapped two distinct regions on chromosome 11p15.5 that are subject to LOH during breast tumor progression and metastasis (6). LOH at region 1 correlated with tumors that contain ductal carcinoma *in situ* suggesting that the loss of a critical gene in this region may be responsible for early events in malignancy. LOH at region 2 correlated with a more aggressive tumor and an ominous outlook for the patient, such as aneuploidy, high S-phase fraction and the presence of metastasis in regional lymph nodes. Although considerable advances have been made in the fine-mapping of chromosome 11p15.5, the tumor suppressor gene(s) encoded by this region have evaded identification.

Integrin-linked kinase (*ILK*) is an intriguing serine/threonine kinase that has been implicated in integrin-, growth-factor- and Wnt-signaling pathways (18). It binds to the cytoplasmic domains of $\beta 1$ and $\beta 3$ integrins and mediates the down-stream signaling events in integrin function (19). Interactions between integrins and their ligands are involved in the regulation of many cellular functions, including embryonic development, cell proliferation, tumor growth and the ability to metastasize (20). In *Drosophila*, the absence of *ILK* function causes defects similar to loss of integrin adhesion and *ILK* mutations cause embryonic lethality and defects in muscle attachment (21). Although *ILK* maps to the commonly deleted chromosome 11p, the potential of this gene in tumor suppression has not been established. We have therefore analyzed the effect of *ILK* expression on the *in vivo* tumor growth and invasion of human mammary carcinoma cells.

B.   BODY:

1. Results:

ILK suppresses tumor formation and metastasis in nude mice

In the last progress report, we reported that we have transfected the ILK gene into the metastatic breast cancer cell line MDA-MB-435 and have isolated four different clones that express different levels of ILK mRNA and protein. We are now testing these cells using a nude mouse metastatic model.

The most stringent experimental test of neoplastic behavior is the ability of injected cells to form tumors in nude mice. Yet not all of the cellular growth properties commonly associated with the cellular state *in vitro* are required for neoplastic growth *in vivo* and vice versa. Therefore, loss of tumorigenecity under expression of *ILK in vivo* would be a critical test to substantiate the growth suppressor function of *ILK*. The mammary carcinoma cell line MDA-MB-435 forms tumors at the site of orthotopic injection, metastasizes in nude mice and closely resembles the course of human breast cancer (22). To investigate whether *ILK* expression affected tumor formation in nude mice, two different *ILK* transfectant clones (TR5-ILK and TR3-ILK) and two vector controls were inoculated into the subaxillary mammary fat pads of 4-6 week old athymic nude mice. Tumors were measured weekly thereafter to assess the growth rate. All MDA-MB-435 vector transfectants were already palpable 7 days after injection. Subsequently, the tumors of vector transfectants grew steadily attaining mean volumes of 3.0 cm^3 (mean ± s.d.) at 15 weeks (Fig. 1A and B). In contrast, only 2 of 12 mice injected with ILK transfectants

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developed tumors. The tumor growth of ILK transfectants was significantly slower than than that of control transfectants ($P < 0.005$, Fisher variance analysis). At sacrifice, (15 weeks) the *ILK* tumors reached a mean volume of only 0.45 cm^3 (mean $\pm$ s.d.) which was significantly smaller than control tumors ($P < 0.001$, Student's *t*-test). Vector transfected MDA-MB-435 cells developed an average of 12-24 lung metastases per mouse (Figure-1C). Additional tumor masses were present in central venous blood vessels, the diaphragm, and lymph nodes of vector transfectants (data not shown). In contrast, with the ILK transfectants, only one of the two animals that developed tumors showed a single metastatic colony in the lung. The presence of additional microscopic metastases in random lung sections was not observed by H&E staining (data not shown). These results clearly demonstrate that the expression of *ILK* in human MDA-MB-435 breast carcinoma cells significantly suppresses tumorigenecity and metastatic ability in athymic nude mice.

2. Methods:
Tumorigenecity and Metastasis Assays

Cells (10^6) were injected into the subaxillary mammary fat pads of 4-6 week-old female athymic nude mice Ncr nu/nu (10-12 mice/group; Taconic Labs, Germantown, NY) as described (52). Mice were maintained under the guidelines of NIH and the Cleveland Clinic Foundation. All protocols were approved and monitered by the the Institutional Animal Care and Use Committee. Food and water were provided *ad libitum*. Tumors were monitered weekly after inoculation. When the mean tumor diameter reached 1.0-1.3 cm, primary tumors were surgically removed under Ketaset-Rompun anesthetic. Mice were then maintained for an additional 4 weeks to allow further growth of lung metastases. After euthanasia, all organs were checked for metastases.

C. CONCLUSIONS:

In previous reports, we have provided evidence that ILK expression is down-regulated in primary breast tumors and in cell lines derived from metastatic breast tumors. We have shown that ILK overexpression inhibits the growth of the highly metastatic breast cancer cell line MDA-MB-435. In addition, ILK overexpression stimulates the levels of the growth suppressing integrin $\alpha 5\beta 1$ and inhibits the levels of $\alpha v\beta 3$, a growth promoting integrin. These innovative studies suggest a novel role for ILK in the etiology of breast cancer. Functional studies in animal models were therefore undertaken to establish ILK as a metastasis suppressor gene. These studies are part of this year's report.

The present study reveals that expression of *ILK* potently suppresses *in vivo* tumorigenecity of the human mammary carcinoma cells. The MDA-MB-435 cells are a model for deficient *ILK* protein expression and transfection of the *ILK* gene is designed to restore this deficiency. As shown in the last year's report, the growth suppression activity requires a functional *ILK* protein, since expression of wild-type *ILK*, but not the ankyrin repeat or the catalytic domain mutants, resulted in growth suppression of MDA-MB-435 cells. These results suggest a possible role for *ILK* in the suppression of tumor growth and metastasis and directly implicate its loss in processes regulating the malignant phenotype in human breast cancer. *ILK* seems to play a dual role in the MDA-MB-435 model system. First, it regulates cell-cycle progression at the G1/S boundary and second, it modulates the levels of integrins, transmembrane receptors that have been shown to regulate growth, differentiation and invasiveness of cells. During this process, the neoplastic cells cease to proliferate and lose their ability to migrate through vitronectin membranes and to induce tumor growth and metastasis in nude mice (Figure-1).

D. KEY RESEARCH ACCOMPLISHMENTS:

- Chromosome 11 harbors a breast cancer metastasis suppressor gene
- Integrin linked kinase (ILK) is a key candidate gene that maps to this region
- ILK expression is downregulated in breast carcinomas that metastasize
- ILK expression inhibits the growth of the metastatic breast cancer cell line MDA-MB-435 both in vitro and in vivo.

These data suggest that ILK functions as a metastasis suppressor gene in breast cancer

E. REPORTABLE OUTCOMES:

- These results are being prepared as a manuscript for publication.

F.   REFERENCES:

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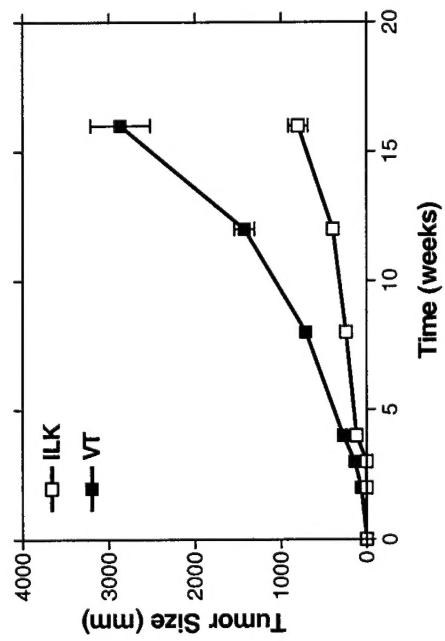
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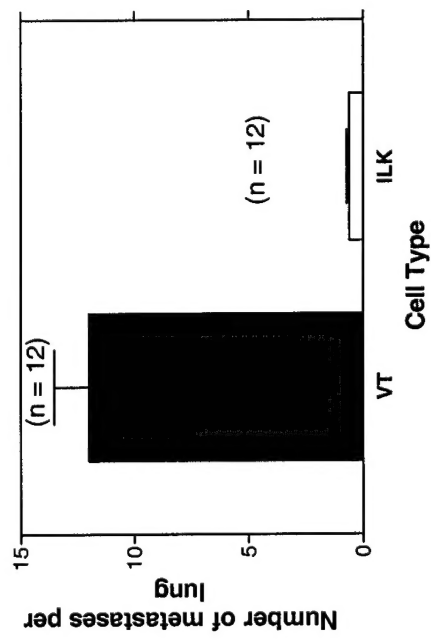
FIGURE LEGEND:
Figure-1: (A) In vivo tumor growth of *ILK* transfected (-□-) and vector transfected (-■-) MDA-MB-435 cells in mammary fat pads of athymic nude mice. Each point represents the mean ± SE of tumors. (B) Five x 10^5 cells of *ILK* transfected (top panel) or vector transfected (bottom panel) MDA-MB-435 cells were injected s.c. into the mammary fat pad area below the nipple. Tumors were allowed to grow for 15 weeks at which time the mice were photographed and sacrificed. (C) Lung colony formation in athymic nude mice injected with vector transfected (VT) or *ILK* transfected *(ILK)* MDA-MB-435 cells. Bars represent S.E.

APPENDIX

A.

Tumor Size (mm)

4000
3000
2000
1000
0

0   5   10   15   20
Time (weeks)

ILK
VT

B.

C.

Number of metastases per lung

15
10
5
0

(n = 12)
(n = 12)

VT   ILK
Cell Type